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In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum

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SUMMARY

IgG anti-filaggrin autoantibodies (AFA) are the most specific serological markers of rheumatoid arthritis (RA). They include the so-called 'anti-keratin antibodies' (AKA) and anti-perinuclear factor (APF), and recognize human epidermal filaggrin and other (pro)filaggrin-related proteins of various epithelial tissues. In this study we demonstrate that AFA are produced in rheumatoid synovial joints. In 31 RA patients, AFA levels were assayed at equal IgG concentrations in paired synovial fluids (SF) and sera. AFA titre-like values determined by indirect immunofluorescence and immunoblotting and AFA concentrations determined by ELISA were non-significantly different in serum and SF, clearly indicating that AFA are not concentrated in SF. In contrast, we demonstrated that AFA are enriched in RA synovial membranes, since the ELISA-determined AFA in low ionic-strength extracts of synovial tissue from four RA patients represented a 7·5-fold higher proportion of total IgG than in paired sera. When small synovial tissue explants from RA patients were cultured for a period of 5 weeks, the profile of IgG and AFA released in the culture supernatants was first consistent with passive diffusion of the tissue-infiltrating IgG (including AFA) over the first day of culture, then with a *de novo* synthesis of IgG and AFA. Therefore, AFA-secreting plasma cells are present in the synovial tissue of RA patients and AFA can represent a significant proportion of the IgG secreted within the rheumatoid pannus.

Keywords rheumatoid arthritis AKA anti-filaggrin autoantibodies synovial membrane B cells

INTRODUCTION

Rheumatoid arthritis (RA) is the most frequent human autoimmune disease. It is characterized by the formation in synovial membranes of an inflammatory and invasive tissue, the rheumatoid pannus that leads to the destruction of synovial joints. The mononuclear cell infiltrate of the pannus is largely composed of immunoreactive cells that engage in multiple interactions leading to synthesis of immunoglobulins [1–3] and mediators of cellular immunity [4]. B cells at various stages of differentiation form an important part of the pannus [5–7]. Some molecular evidence that B cells are specifically activated and clonally expanded in the synovium has been reported [8–10]. Moreover, the germinal centre-like structures of the pannus have been described to contain follicular dendritic cells, $CD4^+$ T cells and activated B cells exhibiting isotype switching and somatic hypermutation [7,10]. Lastly, synthesis of IgM, IgG and IgA by plasma cells of the rheumatoid synovial membranes has been demonstrated [5,11].

A further hallmark of B cell involvement in the pathophysiology of RA is also the presence of various serum antibodies directed to self antigens. Among them, rheumatoid factors (RF) have been the most extensively studied [12]. Even if not highly specific for RA, IgM RF serves as a criterion in the classification of RA [13]. Numerous other serum autoantibodies such as those directed to collagen type II [14,15], histones [16], RA33 antigen [17], Sa antigen [18], calpastatin [19,20], chondrocyte [21] or synovial membrane proteins [22], and cytoskeletal components [23] have been described in RA. Since their description by Young *et al*. [24], the so-called 'anti-keratin antibodies' (AKA), serum IgG labelling the stratum corneum of rat oesophagus epithelium, detected by indirect immunofluorescence (IIF), have been largely demonstrated to be the autoantibodies most specifically associated

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with RA. They are therefore increasingly used for the diagnosis of the disease [25–27]. We demonstrated that 'AKA' are directed to three non-cytokeratin late differentiation proteins of the rat oesophagus epithelium [28]. The antigen recognized was shown to be also present in the stratum corneum of human epidermis [29,30] and was identified as a neutral/acidic isoform of filaggrin, a late-differentiation protein involved in the aggregation of cytokeratin intermediate filaments [31]. In 1964, Nienhuis & Mandema [32] described anti-perinuclear factor (APF) as serum antibodies that decorate by IIF perinuclear granules in the superficial cells of the human buccal epithelium. Numerous studies of this antibody during the last 34 years have confirmed its close association with RA and therefore its diagnostic utility (for a review see [25]). We recently showed that APF detects filaggrin-related proteins of buccal epithelial cells and we provided direct immunological evidence of the large identity between 'AKA' and APF, which we consequently proposed to name anti-filaggrin autoantibodies (AFA) [33]. Recently, we reported that the various filaggrin-related epithelial proteins targeted by AFA are deiminated, their arginine residues being transformed into citrullines [34]. Moreover, we and others showed that the citrulline residues are an essential constituent of the (pro)filaggrin epitopes recognized by AFA [34,35].

Several arguments seem to indicate that AFA are involved in the pathogenesis of RA. Indeed, in addition to their close association with the disease, they have been shown to appear early and even to precede the clinical symptoms [36,37]. Moreover, their presence and titre are related to disease activity and severity [25,26,29,30,38]. However, deiminated (pro)filaggrins cannot be considered as the autoantigen that drives the anti-filaggrin response, since squamous epithelia are not a target of rheumatoid inflammation and (pro)filaggrins are not considered to be expressed in articular tissues. To date, the existence of an articular target for AFA remains to be demonstrated.

AKA and APF had been previously detected in the synovial fluid (SF) of rheumatoid patients [29,39–41] but were never searched for in their synovial tissue. Similarly, the plasma cells of the rheumatoid pannus have never been shown to be able to secrete AFA.

In the present study we investigated whether AFA are more concentrated in the SF and in the pannus interstitium than in the serum, and whether they are locally produced in the rheumatoid pannus. The AFA titre was determined in SF and in extracts of rheumatoid synovial membranes from RA patients and compared with the AFA titre in the paired sera. Production of IgG and AFA by rheumatoid synovial tissue was studied by culturing fragments of the tissue.

PATIENTS AND METHODS

Patient sera, synovial fluids and tissues

Biological samples were obtained from 81 patients classified as suffering from RA according to the criteria of the American Rheumatism Association [13]. Paired samples of SF and serum were concomitantly obtained from 31 patients with an effusion of the knee joint. SF was taken by needle puncture. It was centrifuged and the supernatant collected. The supernatants and the sera were stored at −80°C until assayed. Paired samples of synovial tissue and serum were concomitantly obtained from five other patients, numbered 1–5, who were undergoing surgery for wrist or elbow synovectomy. The synovial tissues were processed immediately after surgery. The joint capsule and articular cartilage were trimmed off, then multiple samples from the macroscopically different regions of the synovium were chosen and either processed for histology or snap-frozen in isopentane precooled in liquid nitrogen and stored at −80°C, or prepared for culture. The sera from the other 45 patients were used for purification of AFA (see below).

Histology

Sections of fixed, paraffin-embedded synovial tissue fragments were stained with haematoxylin and eosin (H–E) and microscopically examined. Histopathological features were recorded and the percentage of plasma cells among mononucleated leucocytes was estimated in 10 randomly chosen high-power (× 40) microscopic fields.

Culture of synovial membranes

For patients 1, 3 and 5, fresh synovial tissue was disrupted in 24 small pieces of about 2 mm^3 which were immediately transferred into 24-well plates (one piece per well) filled with 2 ml/well of RPMI 1640-glutamax supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin (all from Life Technologies, Paisley, UK). The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere. To assess the passive elution of IgG infiltrating the tissue, snap-frozen fragments were comparatively analysed by direct immunofluorescence (see below) before (two fragments) and after (two fragments) a 24-h period of culture. At the same times, four (2 × 2) fragments were also histologically analysed as described above. The 16 remaining tissue fragments were kept in culture for 34 days, the medium being renewed on days 1, 6, 13, 20 and 27. The culture media were centrifuged and supernatants were kept at −80°C until assayed.

Direct immunofluorescence on synovial tissue

Air-dried cryosections of synovial tissue fragments were rehydrated for 15 min in PBS, then incubated for 30 min at 37°C with FITC-labelled goat Fab fragments to human γ heavy chain of immunoglobulin diluted to 1:50 in PBS (Southern Biotech Inc., Birmingham, AL). After one wash in PBS containing 0·05% Tween 20 and one wash in PBS alone, the slides were mounted with Fluoprep medium (BioMérieux, Lyon, France) and observed with a BH2 microscope (Olympus Corp., Tokyo, Japan) with ultra-violet epi-illumination.

Synovial extract preparation

For patients 1, 2, 3 and 4, frozen synovial tissue fragments were weighed, then extracted five times consecutively for 30 s with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at top speed, with 3 ml per gram of tissue of the following ice-cold buffer: 40 mM Tris–HCl, pH 7·4, containing 150 mM NaCl, 10 mM EDTA, 0·02% sodium azide, 2 μg/ml aprotinin (Sigma Chemical Co, St Louis, MO) and 1 mM PMSF (Sigma). After each extraction, the homogenates were centrifuged at 4°C for 20 min at 15 000 ***g*** and the supernatants—identified as low ionic-strength extracts—collected, then stored at −30°C until assayed.

AFA purification

AFA were purified from the sera of 45 RA patients selected for their high AFA titre, as evaluated by IIF on rat oesophagus cryosections. They were shown to react only with the neutral/acidic human epidermis filaggrin by immunoblotting when tested on an enriched fraction of this protein ([27,31] and below).

Purification was performed by pooling 1 ml of each serum diluted to 1:2 in PBS, then loading onto a 5-ml NHS-HiTrap column (Pharmacia, Uppsala, Sweden) coupled with 3 mg of neutral/acidic filaggrin extracted and enriched as previously described [31]. After a 3-h incubation at room temperature, the column was washed with 5 volumes of 1 M NaCl, 10 mM phosphate buffer pH 7·4 and 5 volumes of PBS. Bound antibodies were eluted with 0·2 M glycine–HCl pH 2·5 and immediately neutralized by addition of 2 M Tris. The pooled eluates had a protein concentration of 50 μg/ml and were slightly contaminated by serum albumin. Therefore, the AFA were further purified and concentrated in a protein-G affinity column (HiTrap G 1 ml; Pharmacia). The column was washed and the antibodies eluted as described above. Six milligrams of AFA were obtained. As checked by SDS–PAGE and immunoblotting on filaggrin-enriched epidermal extracts, they were pure and specific for human neutral/acidic filaggrin.

IgG assays

Single radial immunodiffusion. The IgG concentration of sera and SF was measured using a commercially available single radial immunodiffusion kit (Nor-Partigen IgG-HC; Behring, Marburg, Germany). All determinations were performed at least in duplicate.

Sandwich ELISA. The IgG concentration of synovial extracts and culture supernatants was determined using sandwich ELISA. Microtitration plates (Nunc Maxisorp, Life Technologies) were coated overnight at 4°C with 100 μl per well of affinity-purified goat antibodies to human IgG(H + L) (Biosys, Compiègne, France) at 5 μg/ml in PBS. Coated wells were incubated with 100 μl of diluted serum (1:80 000–1:640 000), synovial tissue extract (1:5–1:32 000) or culture supernatant (1:1–1:100), then with 100 μl of goat antibodies to human IgG(γ) conjugated to peroxidase (Southern Biotech) diluted to 1:5000. Incubation times were 1 h at 37°C and each incubation was followed by three washes. PBS containing 0·5% (w/v) teleostean gelatin (Sigma) and 0·05% (v/v) Tween-20 (Pierce, Rockford, IL) was used as sample-dilution and washing buffer. Peroxidase activity was revealed with ortho-phenylenediamine (2 mg/ml; Sigma)–H_2O_2 (0·03%, Sigma). The reaction was stopped with 4 M H_2SO_4 and the optical density (OD) at 492 nm was measured with an ELISA spectrophotometer (Multiskan RC; Labsystems, Life Sciences International S.A., Cergy Pontoise, France). A dilution series of purified human IgG (Jackson ImmunoResearch Labs, West Grove, PA) from 500 to 7·8 ng/ml, included in each ELISA plate, was used as a standard. A log/logit linearization of the standard curve was performed to extrapolate IgG concentrations from ODs. All determinations were performed at least in duplicate.

Assays of anti-filaggrin autoantibodies

Indirect immunofluorescence assay of AKA. The so-called 'AKA' in sera and SF were semiquantitatively titrated by IIF on rat oesophagus epithelium cryosections as previously described [26]. The sera were assayed diluted to 1:10. The SF were diluted so that they reach the same final IgG concentration as the paired serum diluted to 1:10.

Immunoblotting assay of antibodies to human epidermis filaggrin. A detergent extract of human epidermis was obtained by homogenization in 40 mM Tris–HCl pH 7·4, 150 mM NaCl, 10 mM EDTA, 0·5% Nonidet P-40, 0·1% sodium azide, and 0·5 mM PMSF. After centrifugation, the proteins in the supernatant were precipitated with absolute ethanol, recovered by centrifugation at 15 000 ***g*** for 15 min, and resuspended in water after 30 min drying at 80°C. The suspension was centrifuged to obtain neutral/acidic filaggrin-enriched epidermis extracts. In such $\geq$ 80% pure preparations, the neutral/acidic pI of filaggrin is a consequence of its deimination by a peptidyl arginine deiminase [34]. Semiquantitative titration of antibodies to human epidermis filaggrin was performed by immunoblotting on the neutral/acidic filaggrin-enriched epidermis extracts as recently reported [27]. The sera were assayed diluted to 1:30. The SF were diluted with regard to their IgG concentration to obtain the same final IgG concentration as the paired serum diluted to 1:30. The culture supernatants of synovial tissue fragments were assayed using the same protocol except that peroxidase activity was visualized using ECL Western blotting reagents (Amersham Int., Aylesbury, UK).

ELISA of antibodies to human epidermis filaggrin. Human neutral/acidic filaggrin was affinity-purified to homogeneity from an enriched human epidermal extract, with a pool of the four mouse MoAbs AHF-1, -2, -3 and -7, directed to different epitopes of human filaggrin [42], as previously described [33]. Microtitration plates (Nunc Maxisorp) were coated overnight at 4°C with 100 μl per well of purified neutral/acidic filaggrin at a concentration of 2·5 μg/ml in PBS. The plates were then blocked for 30 min at 37°C with 200 μl of 2·5% (w/v) teleostean gelatin and 0·05% (v/v) Tween-20 in PBS. Coated wells were incubated with 100 μl of diluted sera (1:50–1:1600), SF (1:30–1:1600), synovial extracts (1:2–1:100) or culture supernatants (1:1–1:10), then with 100 μl of goat antibodies to human IgG(γ) conjugated to peroxidase (Southern Biotech), diluted to 1:2000. Incubation times, buffers, washings and colour development were as in the sandwich ELISA described above. A pool of 13 RA sera with very high AFA titres was assayed comparatively with the purified AFA. The immunoreactivity of the pool diluted to 1:100 was equivalent to that of AFA at 16 μg/ml. A dilution series of the pool (1:100–1:12 800), corresponding to 16–0·125 μg/ml of AFA, was included in each ELISA plate and used as a standard. Data processing was as mentioned above. Sample concentrations below the lower standard point (0·125 μg/ml) were considered as 0, and concentrations $>$ 4 μg/ml led to re-assay at higher dilutions. All determinations were performed at least in duplicate.

Statistical analysis

Means are given $\pm$ s.e.m. Correlations were sought by computing Spearman's rank correlation coefficient. The Wilcoxon matched pairs test was used to compare IgG and AFA levels between sera and SF. These tests were considered significant when $P < 0{\cdot}01$.

RESULTS

Comparison of AFA titres and concentrations in sera and SF of RA patients

IgG and AFA concentrations were determined in paired SF and sera from 31 RA patients with knee effusions. IgG concentrations (Fig. 1a), measured by single radial immunodiffusion, ranged from 6·9 to 22·8 mg/ml in the sera (mean 14·2 $\pm$ 0·7 mg/ml) and from 3·6 to 18·2 mg/ml in the SF (mean 8·9 $\pm$ 0·7 mg/ml). The IgG concentration in the group of sera was significantly higher than that of the SF group ($P < 0{\cdot}00001$), the ratios of serum IgG to SF IgG ranged from 1 to 3. The AFA titres and concentrations (Fig. 1b) were determined in serum and SF pairs by IIF, immunoblotting and ELISA for equal concentrations of IgG.

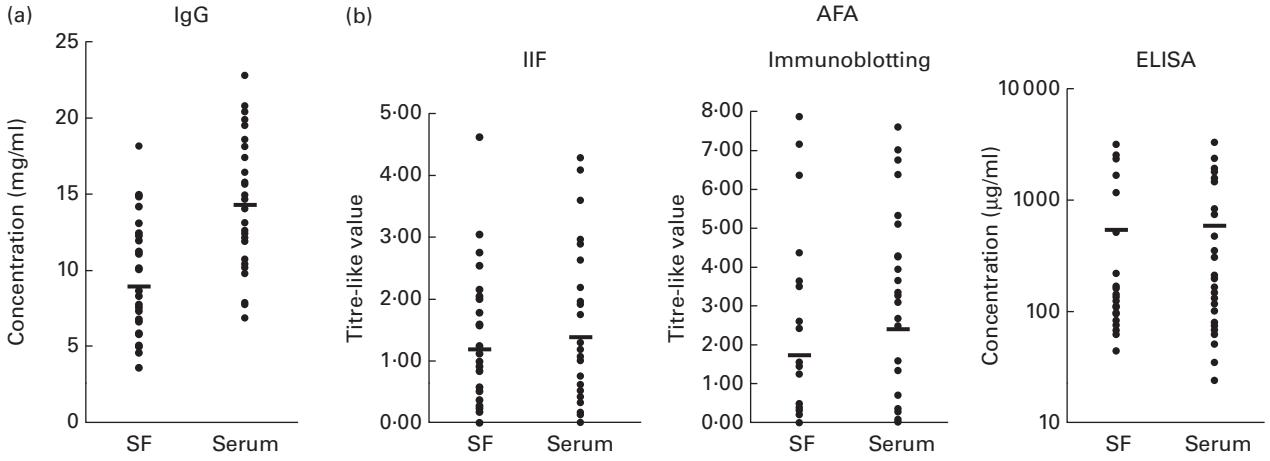

Fig. 1. Synovial fluid (SF) and serum levels of IgG and anti-filaggrin autoantibodies (AFA) in 31 rheumatoid arthritis (RA) patients. IgG (a) and AFA (b) levels were determined in paired SF and sera obtained from 31 RA patients. IgG concentrations were measured by single radial immunodiffusion. AFA titre-like values were determined by indirect immunofluorescence (IIF) on rat oesophagus cryosections and by immunoblotting on neutral/acidic isoform of human filaggrin. Serum and SF were assayed at the same IgG concentration. AFA concentrations were measured by ELISA using purified neutral/acidic isoform of human filaggrin as coated antigen. Mean values are represented as horizontal bars. The mean IgG concentration was significantly higher in the sera than in the SF ($P < 0{\cdot}00001$). The means of AFA in serum and SF were not significantly different whatever the method of assay.

Table 1. Proportion of anti-filaggrin autoantibodies (AFA) among IgG in the serum and synovial tissue of rheumatoid arthritis (RA) patients*

	Serum			Synovial extract†			
Patient	IgG (mg/ml)	AFA (μg/ml)	AFA/IgG (%)	IgG (mg/g)	AFA (μg/g)	AFA/IgG (%)	Syn/ser ratio (AFA/IgG)‡
1	10·51	33·50	0·32	1·46	53·20	3·65	11·46
2	10·75	41·30	0·38	1·21	28·60	2·36	6·13
3	5·55	42·30	0·76	2·37	67·70	2·85	3·75
4	14·59	90·00	0·62	4·75	256·20	5·39	8·75
Mean ± s.e.m.	10·35 ± 1·85	51·78 ± 12·89	0·52 ± 0·10	2·45 ± 0·81	101·43 ± 52·22	3·56 ± 0·67	7·52 ± 1·66

*IgG and AFA concentrations were determined by ELISA in paired sera and synovial extracts of four RA patients.

†Each synovial tissue fragment was weighed, then extracted five times in a low ionic-strength buffer. The antibody concentrations were measured in each extraction supernatant. For each fragment, the sum of amounts calculated in the five consecutive extracts is expressed as mg of IgG or μg of AFA per gram of wet synovial tissue.

‡The ratios between the proportion of AFA to IgG in the synovial membrane (syn) and the serum (ser) were calculated according to the formula:([AFA] in synovium:[IgG] in synovium):([AFA] in serum:[IgG] in serum). For all patients, AFA represent a higher proportion of total IgG in the synovial tissue extract than in the paired serum.

The correlation coefficients between the concentrations determined by ELISA and the titres obtained by IIF and immunoblotting were 0·62 and 0·89, respectively. The correlation coefficient between IIF and immunoblotting titres was 0·63, which was consistent with our previous report [27]. The three correlations were highly significant ($P < 0{\cdot}001$). The AFA titres and concentrations were not significantly different between the group of sera and the group of SF, whatever the detection method. These results indicate that the proportion of AFA among total IgG was not different in SF and serum, and thus that AFA were not concentrated in the former compartment.

Comparison of AFA concentrations in sera and synovial tissues of RA patients

IgG and AFA concentrations in the serum and in the low ionic-strength extracts of synovial tissue from four patients with RA were determined by ELISA (Table 1). The patients had an active synovitis, as judged by histological analysis of the tissue where heavy infiltrates of mononuclear leucocytes and thickening of the synovial lining layer were observed (data not shown). The ratio of AFA to total IgG in the synovial extracts and in the sera were compared. In the sera, the mean IgG concentration was 10·35 ± 1·85 mg/ml. Patients 1, 2 and 4 showed high IgG concentrations, only patient 3 showed a low concentration of 5·55 mg/ml. The mean AFA concentration in the sera was 51·78 ± 12·89 μg/ml. The mean AFA/IgG ratio was 0·52 ± 0·10%, the ratio of patient 3 being in the same range as the three other ratios. In the synovial tissue extracts, the antibody concentrations were expressed as mg of IgG or μg of AFA per gram of wet synovial tissue. The mean IgG concentration was 2·45 ± 0·81 mg/g, the mean AFA concentration was 101·43 ± 52·22 μg/g and the mean ratio AFA/IgG was 3·56 ± 0·67%. The relative concentration ratios of AFA between the synovium and serum ranged from 3·75 to 11·46 (mean 7·52 ± 1·66). Therefore,

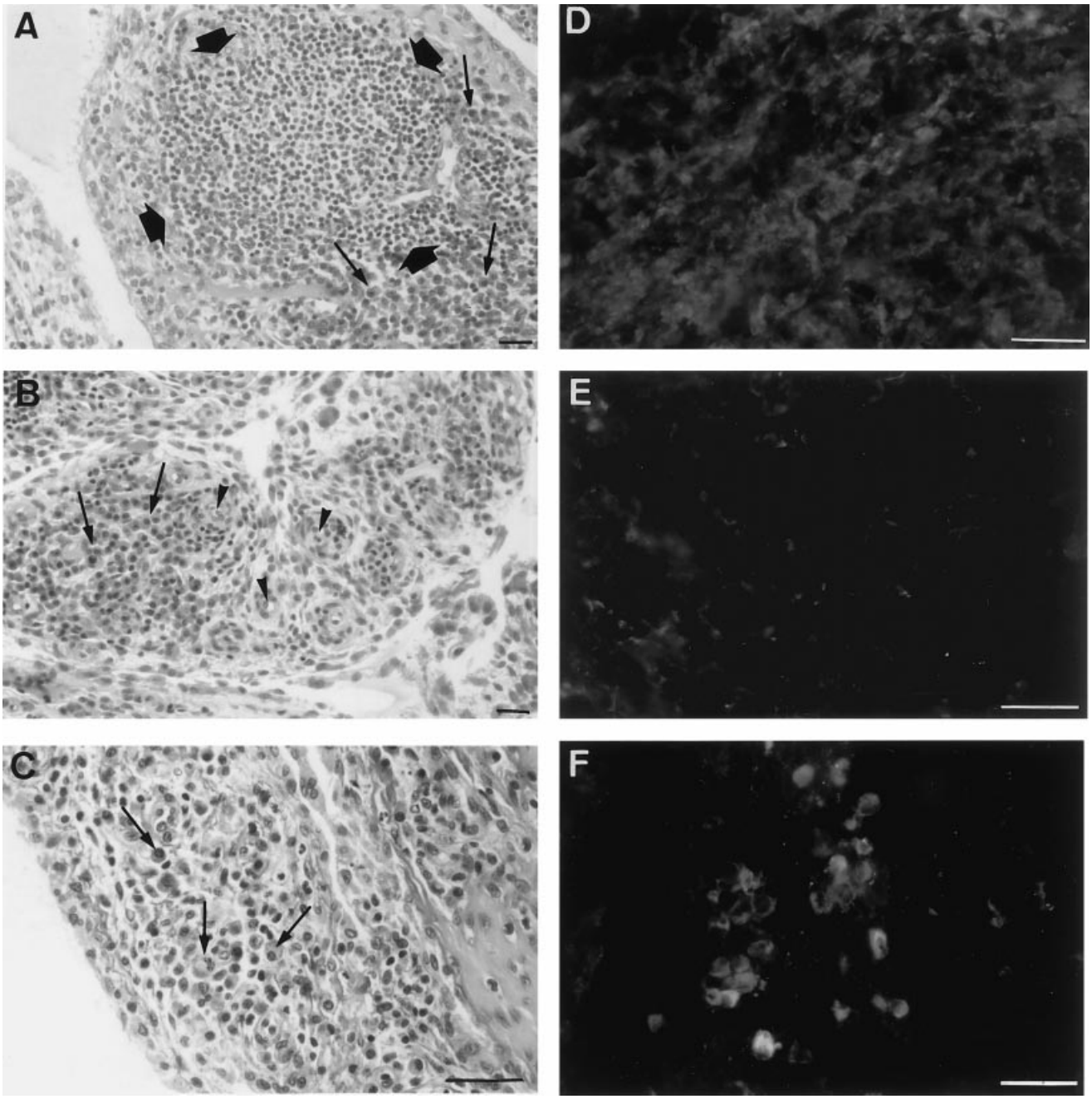

Fig. 2. Detection of plasma cells and infiltrating IgG in synovial tissue from rheumatoid arthritis (RA) patients. Paraffin sections of synovial tissue before (A,C) and after (B) 24 h of culture were observed after staining with haematoxylin and eosin. Plasma cells are identified by their eccentric nuclei, large amounts of cytoplasm and perinuclear halo (thin arrows). In (A) plasma cells surround lymphoid follicles (thick arrows); in (B) they are close to blood vessels (arrowheads); in (C) they are interspersed amongst synovial lining cells. Cryosections of synovial tissue before (D) and after (E,F) 24 h of culture were analysed by immunofluorescence using FITC-labelled Fab fragments to human IgG. Before culture the synovial tissue fragments were heavily infiltrated with IgG (D). After 24 h of culture, the level of IgG infiltration was dramatically decreased (E) and clusters of cells exhibiting the nuclear/cytoplasmic ratio typical of plasma cells became distinguishable (F). Bars = 50 μm.

in the four patients, AFA represented a higher proportion of IgG in the synovial tissue extract than in the serum.

Detection of plasma cells in synovial tissues

The histology of synovial tissues from RA patients 1–5 was thoroughly analysed on H–E-stained sections before and after 24 h of culture (Fig. 2). Classical histological lesions were detected in all the tissue samples: hyperplasia of the synovial lining layer, and intense mononuclear cell infiltration arranged in nodular aggregates with germinal centres. Plasma cells constituted a high proportion (mean 42 ± 16%) of the mononuclear cells. Many of them surrounded the lymphoid follicles (Fig. 2A) and

were in close proximity to blood vessels (Fig. 2B), or intermixed with synovial lining cells (Fig. 2C).

The degree of IgG infiltration of the tissue was evaluated by direct immunofluorescence before and after 24 h of culture (Fig. 2). Before culture (Fig. 2D), intense and diffuse fluorescence was observed all over the sections, showing a high concentration of interstitial IgG. After 24 h of culture (Fig. 2E), fluorescence diminished drastically, indicating the release of interstitial IgG. By contrast, numerous clusters of cells, with an intense patchy and granular cytoplasmic fluorescence, exhibiting the morphological characteristics of plasma cells, were observed (Fig. 2F). These IgG-producing plasma cells were found to be scattered in the tissue.

In vitro *production of AFA by synovial plasma cells*

To test whether AFA were produced by synovial plasma cells, synovial tissue fragments (16 fragments of approximately 2 mm^3) from three RA patients (numbers 1, 3 and 5) were cultured over a period of 34 days. Patients 1 and 3 had significant titres of serum AFA whereas patient 5 had no detectable serum AFA. IgG were assayed by ELISA and AFA by ELISA and immunoblotting in the supernatants from days 1, 6, 13, 20, 27 and 34. The mean IgG concentrations in day 1 supernatants were 2·21 μg/ml, 4·77 μg/ml and 1·33 μg/ml for patients 1, 3 and 5, respectively. As shown above by direct immunofluorescence, these IgG mainly corresponded to the release of tissue-infiltrating antibodies. The mean amounts of IgG and AFA secreted by the fragments from days 2 to 34 were summed, only the fragments producing detectable IgG being taken into account. On day 34, the summed amounts of IgG were 17·20 μg, 34·08 μg and 62·16 μg and the cumulated AFA amounts were 1·28 μg, 0·80 μg and 0 μg for patient 1, 3 and 5, respectively. Therefore, the AFA/IgG ratios were 7·48%, 2·32% and 0%, respectively. These data indicate the existence of an initial quick release of interstitial IgG followed by *de novo* synthesis of IgG, including AFA. The AFA immunoblotting reactivity of the culture supernatants also reflected the release then the *de novo* synthesis of these antibodies (Fig. 3).

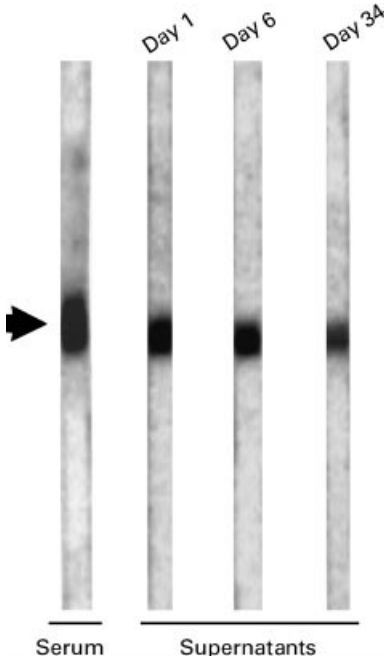

Fig. 3. Immunoblotting detection of anti-filaggrin autoantibodies (AFA) produced *in vitro* by synovial tissue explants. Sixteen 2-mm^3 synovial tissue explants from a rheumatoid arthritis (RA) patient (number 3) were cultured separately for a period of 34 days. An immunoblotting reactivity to the neutral/acidic isoform of human filaggrin (arrowhead) is observed in the serum of the patient and in culture supernatants of the synovial explants at days 1, 6 and 34.

DISCUSSION

Autoimmune reaction and chronic inflammation in the synovial tissue are the main pathophysiological phenomena occurring in RA joints [1]. The view is widely shared that T cell reactivity is the driving force behind rheumatoid inflammation [4]. One manifestation of the immunological reactivity in rheumatoid synovitis is also the presence of infiltrating plasma cells that secrete immunoglobulins into the synovial tissue [1,2,5]. Indeed, in RA, synovial joint immunoglobulin production may reach levels equivalent to those in the spleen [1] and is thought to participate in the pathogenesis of rheumatoid tissue injury [43]. A number of investigators have demonstrated that self-reactive antibodies can be produced in the inflamed joints of RA patients [1,2,5,11,44–48]. The number of RF-producing precursors was shown to be higher in the rheumatoid SF than in the blood [49], and the population of B cells specific for the IgG Fc fragment has been shown to be expanded locally within the affected joints of RA patients [50]. The aim of our study was to determine whether AFA are also produced within the inflamed synovial joints of RA patients. One of our approaches was to evaluate the AFA content of low ionic-strength extracts of synovial tissue samples from patients with active RA synovitis and significant titres of serum AFA. The results obtained clearly indicate that AFA were present in all four of the rheumatoid synovial membranes analysed. Moreover, when AFA and IgG concentrations were compared between the synovial tissue extracts and paired sera, the relative concentration ratio of AFA to IgG between the synovium and serum ranged from 11·46 to 3·75, showing that for all the RA patients analysed, AFA represented a substantially higher proportion of IgG in the synovial tissue extract than in the paired serum. This suggests that AFA synthesis takes place within the rheumatoid synovial joint. Moreover, when the insoluble material from the different synovial membranes obtained after five extractions in low ionic-strength buffer was re-extracted using a high urea concentration, measurable amounts of AFA were found in the supernatants (data not shown). Therefore, the AFA levels measured in the low ionic-strength extracts are an underestimate of the total amounts present in the synovial tissue.

T lymphocytes are often considered to be the predominant population of mononuclear leucocytes that infiltrate the synovium [51]. However, Brown *et al*. [52] assessed the distribution of plasma cells in RA synovial tissue and showed that they constitute the most abundant infiltrating mononuclear cell population, and are metabolically very active. In agreement with these data and with a previous report in which plasma cells were shown to compose 25–50% of all inflammatory cells in synovial tissue sections [53], we also found that they constitute the predominant population of infiltrating mononuclear leucocytes in all the rheumatoid synovial tissues studied. They were not confined to a particular location such as plasma cell-rich areas [54] but were present throughout synovial villi, distributed amongst and below

synovial lining cells, in mononuclear cell infiltrates, around small venules and frequently in the blood vessel walls themselves.

To test the hypothesis that a proportion of plasma cells in RA synovial tissue are AFA-secreting cells, fragments of rheumatoid synovial tissues were cultured for a 5-week period and studied for their AFA production. While most authors analysing immunoglobulin production by rheumatoid synovial membranes cultured the tissues after dissociation into single-cell suspensions [45,48,55], we used tissue explants of about 2 mm^3. In these conditions, the tissue architecture and extracellular matrix are minimally disrupted, allowing the integrity of the original inflamed tissue to be preserved. Synovial plasma cells being kept in their *in vivo* microenvironment, one could expect that the antibody secretion measured would give a better indication of pathophysiological reality. Interestingly, as most studies using dissociated cells noted maximal IgG production at around day 7 of culture [45,55], using prolonged culture of tissue fragments we were able to detect IgG production as late as after 5 weeks of culture. A great variation was observed in the measured IgG and AFA concentrations between synovial tissue explants from the same synovial membrane. This may however be explained by the variability in the number of IgG- and AFA-secreting plasma cells present in the different explants, inherent to the histological diversity of the inflamed synovial membranes. Overall, the amounts of secreted IgG and AFA gradually increased over time to reach levels much higher than those observed in the supernatants after the first day of culture, a time when immunofluorescence staining not only indicated the almost total absence of infiltrating IgG but also the presence of IgG-producing plasma cell clusters. This persistence over time of spontaneous IgG and AFA production thus demonstrates the existence of *de novo* synthesis. Taken together, our results show for the first time that rheumatoid synovial membranes are infiltrated by AFA-producing plasma cells. It is noteworthy that the two patients from whom the cultured synovial explants secreted AFA had significant titres of AFA in their serum, whereas the third patient with no AFA secretion had no AFA detectable in the serum.

IgG and AFA concentrations were also measured in the SF of 31 RA patients and compared with the IgG and AFA concentrations in their serum. For almost all patients, the IgG concentration was higher in the serum than in the SF, in agreement with previous studies [41,56]. Consequently, before any AFA detection the SF were diluted so that their IgG concentration equalled the IgG content of the corresponding serum when diluted to be assayed. Whatever the AFA detection method, the mean AFA levels were found to be higher in the serum but the difference between the serum and the SF was not statistically significant. These results indicate that AFA do not represent a higher proportion of IgG in the SF than in the serum. In a comparison of 58 paired samples of RA serum and SF, Youinou *et al*. [39] reported IgG AKA titres in the SF lower than in the corresponding sera in a large majority of samples. Nevertheless, these data were not corrected with regard to the lower IgG concentration in SF. Our results agree with those obtained by Quismorio *et al*. [29] with paired samples from five RA patients after adjusting IgG concentrations in the serum to equal the IgG content of the paired SF. In this study, AKA were undetectable in three cases and presented equal titres in serum and SF in the remaining two cases. Only Kirstein *et al*. [40], studying a series of 16 sample pairs, concluded that AKA constitute a higher percentage of IgG in SF after a questionable correction of the AKA titres to take into account the lower IgG concentration in the SF. Therefore, for RA patients, even though IgG-producing (including AFA-producing) plasma cells are present in the synovial membrane, the concentration of IgG is lower in the SF than in the serum. Moreover, the proportion of AFA was found to be lower in the SF and serum than in the synovial tissue interstitium. This supports the idea that the rheumatoid pannus is a preferential location for synthesis of AFA and that serum AFA, at least partially, come from that source. Apart from the still not documented presence of AFA-secreting plasma cells in the joint space, an important source of the SF AFA may also be the synovial membrane plasma cells, especially those located among synovial lining cells. Accumulation of high levels of AFA in the joint space might therefore be expected but was not observed. Primarily, this could be due to the impermeability of the synoviocytic barrier between synovium and fluid. Alternatively, the AFA may be absorbed onto putative autoantigen(s) that could be exposed in the inflamed joint. Finally, it is conceivable that these autoantibodies may be complexed by locally produced RF and subsequently taken up by activated phagocytic cells either in the pannus or in the inflamed joint space.

In agreement with previous studies [29,39,40], when we measured AFA levels in pairs of serum and SF from 29 non-RA patients, including 18 patients with inflammatory rheumatic diseases and 11 patients with non-inflammatory rheumatic diseases, we found that AFA were undetectable in both the serum and SF of these patients (data not shown). These data indicate that the presence of AFA not only in the serum but also in the SF is highly specific for RA. Consequently, even if the presence of AFA-producing plasma cells in the joints of non-RA patients remains to be explored, it is highly improbable. Consistently, in the present study the synovial explants that produced no detectable AFA originated from an RA patient with no detectable AFA in the serum.

To date, the pathophysiological role for AFA in RA has not been demonstrated. However, as mentioned earlier, numerous indirect clinical arguments implicate AFA in RA pathophysiology. The data presented here constitute more evidence suggesting this involvement. Indeed, the high concentration of AFA and the presence of AFA-secreting plasma cells within the main site of immunopathological activity in RA suggest the presence of a target for these antibodies in rheumatoid joints, and raise the possibility that this articular autoantigen may drive the humoral AFA response. Having shown that the filaggrin-related epithelial antigens recognized *in vitro* by AFA are deiminated proteins, and having demonstrated, together with Schellekens *et al*., that citrulline residues are an essential component of the epitopes recognized by these antibodies [34,35], we are now searching for a deiminated AFA-specific autoantigen in the synovial joints of RA patients. Identification of such an articular autoantigen might help to elucidate the mechanism of AFA production and may provide new insights into the pathophysiological mechanisms of the generation and/or perpetuation of chronic synovitis which ultimately leads to cartilage and bone destruction.

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